STUDIES ON BACTERIAL UTILIZATION OF URONIC ACIDS

III. Induction of Oxidative Enzymes in a Marine Isolate[1]

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MacLeod *et al.* (1954) noted that a number of strains of bacteria from the marine environment which can not grow on fresh water media are dependent upon Na^+, K^+, and Mg^{++}, whereas several similar isolates capable of growth on fresh water media have no Na^+ dependence. A subsequent investigation of the effect of these metal ions on the viability in storage and the activity of oxidative systems of a Na^+ dependent strain (Tomlinson and MacLeod, 1957) showed a definite requirement for the three ions for storage and for Na^+ and K^+ for the oxidation of succinate and alanine.

In this laboratory a marine bacterium capable of forming induced enzymes for the oxidation of uronic acids has been isolated from glucuronate enriched marsh mud from Sapelo Island, Georgia. This paper represents a study of the effects of substrate, sea salt, and Na, K, and Mg salts on the induction of the oxidative system.

MATERIALS AND METHODS

Test organism. The physiological characteristics of the microorganism M11 were determined by culturing in differential media prepared with sea water as diluent. These media were sterilized by filtration. Concentrates of media in which agar was required were filtered and added to tempered suspensions of sterile sea water agar. Stock cultures were maintained on sea water nutrient agar slants and all cultures were incubated at 30 C unless otherwise indicated.

Electron photomicrographs of the bacteria were kindly prepared by Dr. N. M. McClung of this Department. Young cells from an agar slant were used.

Respirometry. Bacteria were cultured in sea water nutrient broth, broth supplemented with 0.25 per cent galacturonate, or minimal salts and 0.25 per cent glucuronate in sea water on a rotary shaker at 30 C for 18 hr and harvested by centrifugation. The cells were twice washed with sea water or 0.052 M $MgCl_2$ and suspended in concentrations which gave 5 per cent transmittance in the various suspending media as indicated in the figures. Washing in distilled water inactivated the cells, but 0.052 M $MgCl_2$ as a washing solution prevented cytolysis (Tomlinson and MacLeod, 1957). The oxygen uptake of 2.0 ml of each of the suspensions was measured at 30 C using conventional manometric techniques. The cells were equilibrated for 15 min before the substrate was added. Substrate was 1.0 ml of 0.02 M sodium glucuronate or galacturonate and all systems were buffered at pH 7 with 0.04 M mono- and di-basic sodium phosphates.

Experiments were done as well with cells washed with solutions of $MgCl_2$ and preincubated with 0.02 M glucuronate for 2 hr in solutions of each of the chlorides on a rotary shaker at 30 C. The other salts were added from one or two side arms of the Warburg vessels in concentrations which gave the desired molarity in a volume of 3.0 ml. Further description is given in figure 6.

RESULTS

Characterization of the bacterium. Electron photomicrographs in figure 1, which show the effects of the prolonged suspension of the cells in distilled water during preparation for exposure in the microscope, establish the identity of the bacterium M11 as a pseudomonad by the revelation of polar flagellation. Burkholder and Bornside (1957) obtained morphologically similar isolates from the Sapelo marsh.

Inspection of the results in table 1 indicate that the organism does not have the characteristics of any of the members of the Pseudomonadales described in *Bergey's Manual of Determinative Bacteriology* (1957). Cultures have been

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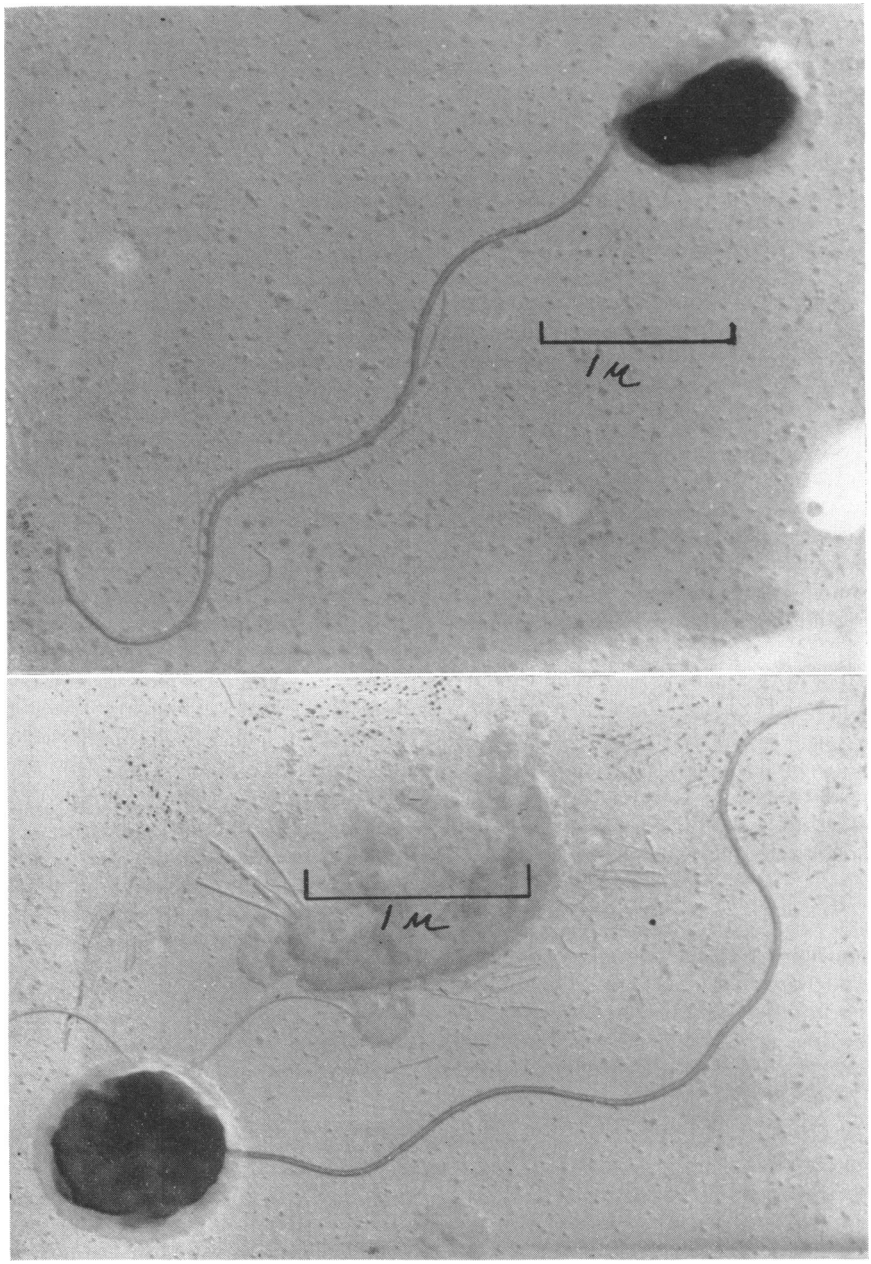

Figure 1. Electron photomicrographs of marine bacterium M11 demonstrating polar flagellation.

submitted to Dr. Einar Leifson of Loyola University of Chicago for investigation of the classification of the isolate.

Effects of substrate and sea salt on induction. The curves in figure 2 indicate a response to glucuronate, by cells grown on galacturonate, which is different than any of those previously reported (Cohen, 1949; Payne and Carlson, 1957). Oxidation of glucuronate during the first hour appears to have been accomplished by nonspecific, galacturonate induced enzymes. Subsequently, more active enzymes were induced in response to glucuronate, and the rate of oxidation of the substrate increased about eightfold.

TABLE 1

Some characteristics of marine bacterium M11

Morphology	Fermentation	Other Metabolic Activity
Colonial:	*Acid produced with:*	Nitrite produced from nitrate.
Circular, smooth, entire, convex, translucent, nonpigmented colonies on agar plates.	L-Arabinose	Indole produced.
	Glucose	Methyl red test positive.
	Fructose	Starch hydrolyzed.
	Galactose	Salicin hydrolyzed.
Abundant, spreading, glistening, viscid, white growth in agar strokes with medium unchanged.	Mannose (±)	Inulin utilized.
	Sucrose	
	Maltose	Alginic acid not hydrolyzed.
	Glycerol	Gelatin not hydrolyzed.
	Mannitol	Hydrogen sulfide not produced.
Cellular:		Urea not hydrolyzed.
Short nonsporogenous, gram-negative rods with single polar flagella.	*Acid not produced with:*	Voges-Proskauer negative.
	D-Arabinose	
	Xylose	*Effect of temperature on growth:*
Average length, 2.5 μ.	Lactose	4 C − 25 C ++
Average diameter, 0.3 μ.	Adonitol	15 C + 30 C +++
	Gas not produced.	20 C + 37 C +
	Acid, no curd in litmus milk, litmus reduced.	Facultative anaerobe.

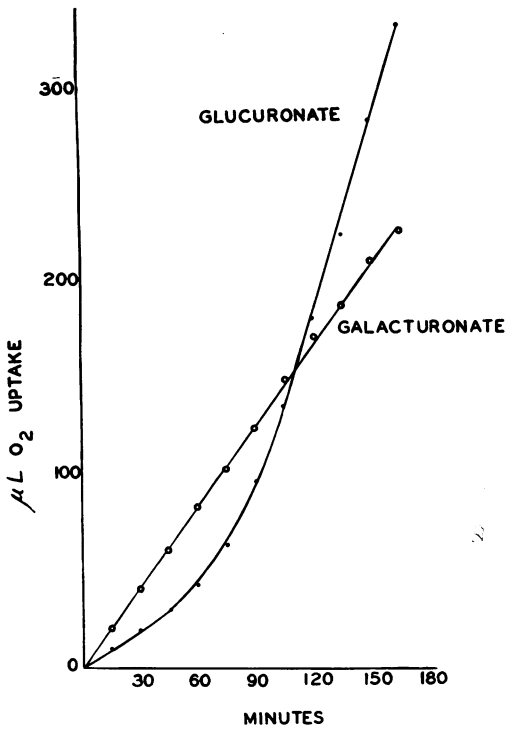

Figure 2. Oxidation of galacturonate and glucuronate by marine bacterium M11 grown on galacturonate broth, washed with sea water and suspended in 3 per cent sea salt water.

Galacturonate was not oxidized by cells grown on broth or glucuronate.

Time required for induction of glucuronate oxidation in resting suspensions of cells grown in sea water broth and washed with sea water was sharply increased by decreasing concentrations of sea salt in the suspending medium (figure 3). The maximum rate of oxidation obtainable varied directly with the concentration of sea salt, and no activity was observed in the absence of salt. To determine if enzymes were induced but inactive in the salt-free system, a concentrated solution of sea salt was tipped into a suspension of cells in distilled water after an incubation period of 45 min. Cells in sea salt water in a simultaneous system were quite active at that time, but induction apparently did not begin in the absence of salts (figure 4). Suspension in distilled water for this period did not inactivate the cells, for introduction of salts permitted induction and oxidation to occur.

Effect of specific salts on induction. Washing in 0.052 M $MgCl_2$ yielded cells which could be induced to good activity in neutralized sea water. Suspension in solutions of the major salts of sea water in various combinations enabled the cells to become active in varying degrees (figure 5). Curves *F* and *E* show that $MgCl_2$, in the absence of Na and K salts, did not support induction of

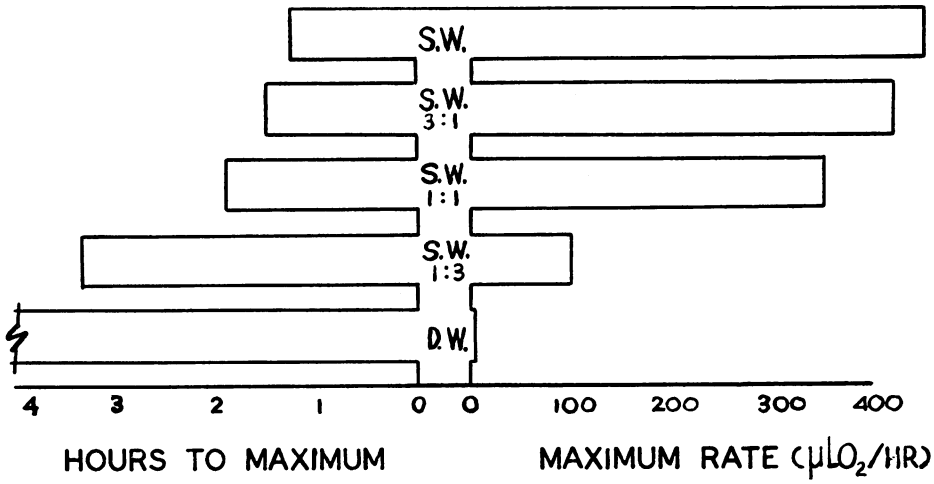

Figure 3. Effect of decreasing concentrations of sea salt on rate of induction and oxidation of glucuronate by marine bacterium M11 grown on broth and washed with sea water.

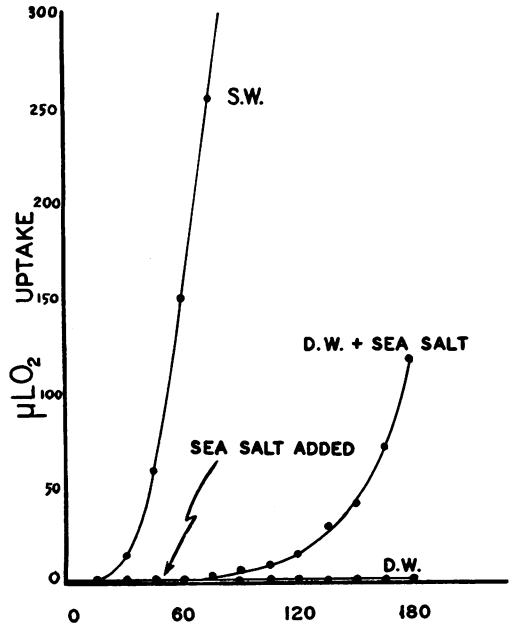

Figure 4. Effect of adding sea salt to a resting suspension of marine bacterium M11 on induction of glucuronate oxidizing enzymes. Side arms in test vessel contained 0.5 ml of 0.01 M sodium glucuronate and 0.5 ml of 18 per cent sea salt.

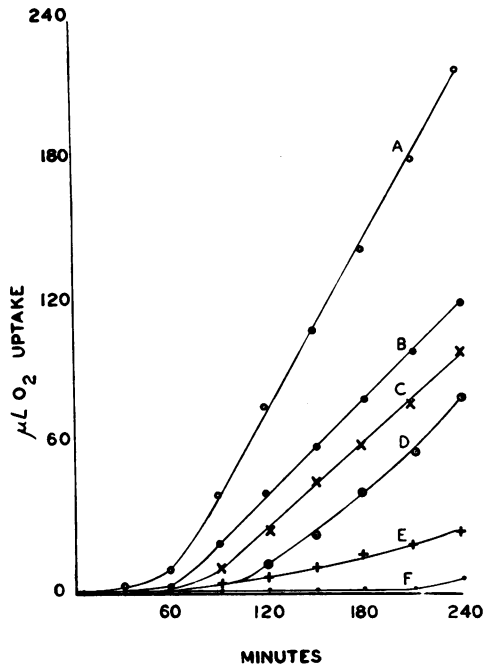

Figure 5. Influence of various combinations of NaCl, KCl, and $MgCl_2$ in the suspending media on the induction of glucuronate oxidizing enzymes in marine bacterium M11. *A*, Neutralized sea water. *B*, NaCl, 0.46 M; KCl, 0.01 M; $MgCl_2$, 0.0245 M; $MgSO_4$, 0.0275 M. *C*, NaCl, 0.46 M; KCl, 0.01 M; $MgCl_2$, 0.052 M. *D*, NaCl, 0.46 M; KCl, 0.01 M. *E*, NaCl, 0.46 M or KCl, 0.01 M (or NaCl and $MgCl_2$ or KCl and $MgCl_2$). *F*, $MgCl_2$, 0.052 M. Endogenous respiration was greatest in sea water and least in the suspensions containing single salts.

activity nor supplement the ability of Na or K salts, in the absence of one or the other, to facilitate induction. Furthermore, curve *E* indicates neither NaCl or KCl alone permits a significant degree of activity. A combination of NaCl and

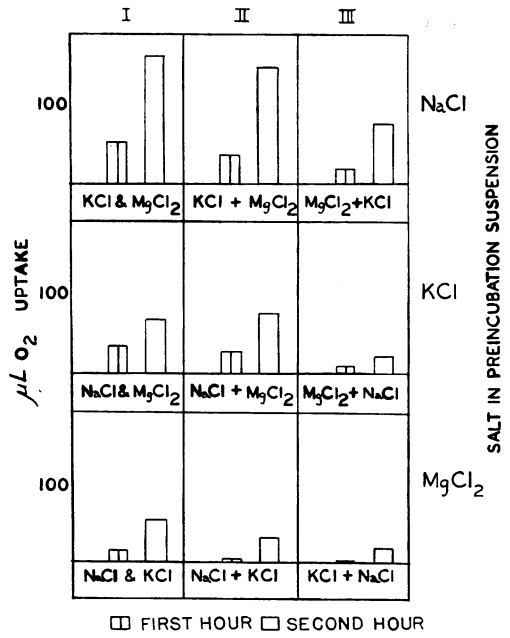

Figure 6. Response to the addition of the other salts of suspensions of marine bacterium M11 preincubated with sodium glucuronate in solutions of 0.46 M NaCl, 0.01 M KCl or 0.052 M $MgCl_2$. In the experiments described in column *I* both salts were added simultaneously, and in those in columns *II* and *III* the second salt was added 30 min after the first. Endogenous respiration was 40 to 50 µL in 2 hr in each system.

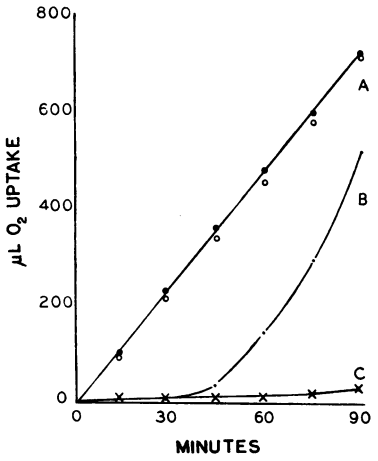

Figure 7. Effect of suspending medium on oxidation of glucuronate by suspensions of marine bacterium M11 grown on glucuronate. *A*, KCl, 0.01 M or KCl and NaCl, 0.46 M. *B*, No salts. *C*, NaCl, 0.46 M.

KCl, however, enabled the cells to be induced to nearly one-third of the activity demonstrable in sea water (curve *D*). This effect was not duplicated by increasing the concentration of KCl to 0.47 M nor by using 0.46 M erythritol in place of NaCl as a presumably inert agent for increasing osmotic pressure. Sucrose, which has been used as an osmotic agent in other studies (Tomlinson and MacLeod, 1957), was rapidly oxidized by these cells. A combination of the three metallic chlorides restored the cells to approximately one-half the activity observed in sea water (curve *C*), and the addition of $MgSO_4$ (with appropriate adjustment in the concentration of $MgCl_2$ to maintain the molarity of Mg^{++}) indicated that $SO_4^{=}$ enhances the rate of induction and activity slightly (curve *B*).

Results obtained by measuring the activity of cells preincubated in solutions of a single salt followed by the addition of the other two, or of one then the other, were revealing with respect to the effect of Na^{+}. Data in figure 6 indicate that preincubation in NaCl yielded cells with good activity. These bacteria were induced, for oxidation was linear from the time KCl was tipped in, and the presence of KCl served to increase the rate of subsequent induction and activity. KCl did not replace the effect of NaCl in preincubation, and $MgCl_2$ alone seemed not to be involved in induction and increased activity beyond its role in preventing cytolysis.

Cells induced to glucuronate in sea water culture seemed nearly indifferent to NaCl but oxidized the substrate rapidly when suspended in solutions containing KCl (figure 7).

DISCUSSION

The results of this and other studies of uronic acid utilization (Cohen, 1949; Payne, 1956; Payne and Carlson, 1957) reveal that glucuronic and galacturonic acids vary from species to species in their ability to induce enzymes which use either acid as a substrate. *Escherichia coli*, *Erwinia carotovora*, *Serratia marcescens*, and isolate M11 are induced by one or the other acid to oxidize its isomer, more or less rapidly than the inducer; but *Shigella flexneri* strain 2a cultured on galacturonate is inactive with glucuronate.

The essentiality of Na and K salts in the metabolism of whole cells of isolate M11 demon-

strated in this study is in agreement with the results of Tomlinson and MacLeod (1957) with another marine bacterium. In addition, the range of influence of the salts in bacterial metabolism has now been shown to include induction of oxidative enzymes. No indication is given of the precise biochemical role of the inorganic ions in these studies. However, it is significant to note that cells preincubated in solutions of NaCl and substrate were induced and became active with the addition of KCl and that those incubated in a solution of both NaCl and KCl were induced to the extent of one-third of the activity demonstrable in sea water. This suggests that NaCl (or probably Na^+) has an indispensable role in the elaboration of the cell's permease system (Cohen and Monod, 1957) and the oxidase system, whereas KCl (or K^+) is involved in the activation of oxidation.

Experiments with bacteria induced in culture to glucuronate support this conclusion, for suspension of the cells in NaCl at sea water concentrations did not permit oxidation. Suspension in a marine level solution of KCl or of KCl and NaCl provided conditions for rapid oxidation. The requirement for a minimal concentration of Na^+ for the oxidation of substrate established by MacLeod *et al.* (1958) is met by the substrate and the dilute buffer in all these experiments. Cells suspended in 0.04 M buffer without added salts show that oxidation in the KCl system was not attributable to enzymes released by cytolysis. Suspensions in the more dilute solution where leakage should be expected did not begin oxidizing at a linear rate for an hour after substrate was tipped in. Activity in the KCl system was linear from the outset.

Considering these results and those of MacLeod's group, it seems likely that many of the bacteria which can grow either in the presence or absence of sea water might be considered to have adapted permeases indifferent to Na^+. On the other hand, strictly terrestrial bacteria might be thought to have evolved further to sensitivity to Na^+.

ACKNOWLEDGMENT

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SUMMARY

Resting cells of a marine psuedomonad, induced in culture to galacturonate utilization and washed with sea water, oxidized glucuronate initially at 50 per cent of the rate on galacturonate. After an incubation of 2 hr, the rate of oxidation of glucuronate increased to approximately three times that of galacturonate. Cells grown on glucuronate did not oxidize galacturonate.

Induction of glucuronate oxidation in resting cells grown in sea water nutrient broth, washed with sea water, and suspended in sea salt water was inhibited by dilution of the sea salt water. Addition of sea salt to cells incubated with substrate in distilled water enabled induction to occur. Washing with 0.052 M $MgCl_2$ provided cells which were inducible to significant activity in neutralized sea water and, to lesser degrees, in solutions of Na, K, and Mg salts.

NaCl and KCl were found to be indispensable to the cells for induction and activity. Supplementing solutions of these salts with $MgCl_2$ and $MgSO_4$ increased the rates of induction and oxidation. In experiments in which cells were preincubated with substrate in solutions of single salts, the dependence of induction on Na^+ and of oxidation on K^+ was demonstrated. Oxidation of glucuronate by resting cells grown on that substance was linear from the time the substrate was added only in systems containing K^+.

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